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Short communication

Genomovar status, virulence markers and genotyping of *Burkholderia cepacia* complex strains isolated from Brazilian cystic fibrosis patients

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Abstract

Burkholderia cepacia complex isolates obtained by microbiological culture of respiratory samples from Brazilian CF patients were studied by *recA* based PCR, screened by specific PCR for virulence markers and genotyped by RAPD. Forty-one isolates of *B. cepacia* complex were identified by culture and confirmation of identity and genomovar determination obtained in 32 isolates, with predominance of *B. cenocepacia* (53.1%). Virulence markers were not consistently found among isolates. Genotyping did not identify identical patterns among different patients. *B. cenocepacia* was the most prevalent *B. cepacia* complex member among our patients, and cross-infection does not seem to occur among them.

Keywords: *Burkholderia cepacia* complex; Cystic fibrosis; Children; Virulence factors; Molecular epidemiology

1. Introduction

The *Burkholderia cepacia* complex has been recognized as a significant pathogen for CF patients since the early 1980s and consists of ten closely related species, also called genomovars [1]. Most specialized centres report a prevalence of *B. cepacia* complex of up to 10% [2–4]. *B. cenocepacia* is a member of the *B. cepacia* complex showing the highest prevalence among CF patients, followed by *B. multivorans*, although regional differences in genomovar distribution have been reported [5].

Highly transmissible strains of the *B. cepacia* complex have been identified in some CF centres, mainly in the United Kingdom and Canada, and genetic markers including cable pilus gene (*cblA*) and an insertion sequence named *B. cepacia* epidemic strain marker (BCESM) are commonly detected in these strains [6,7].

There is little published data regarding prevalence and genomovar distribution of *B. cepacia* complex strains isolated from Brazilian cystic fibrosis patients [8–10]. The Pediatric Pulmonology Unit of Instituto da Criança is one of the referral centres for CF care in Brazil and, although segregation of patients based on respiratory colonization has not been implemented to date, cross-colonization with *P. aeruginosa* strains has not been identified among patients [11]. The objective of the present study was to analyse *B. cepacia* complex strains isolated from CF patients attending our CF centre, by determining genomovar distribution, virulence-related genetic markers and the genotype of these isolates for epidemiological purposes.

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2. Materials and methods

2.1. Patients and samples

Samples identified as belonging to the *B. cepacia* complex in the microbiology laboratory during two periods (regular use of *B. cepacia* complex-selective medium) were studied: period A (September 2000 to April 2001) and period B (June 2003 to June 2004). A total of 140 CF patients attended our unit during these two periods, and the diagnosis of CF was based on international consensus guidelines [12]. Respiratory samples were collected from the patients during routine and unscheduled visits to the outpatient clinic and cultured on selective media, including *B. cepacia* complex-selective medium (Oxoid, Basingstoke, United Kingdom). Plates were incubated at 36 °C for 18 to 96 h.

During the first period of the study (period A), bacteria were identified using the Vitek automated system (bioMérieux Vitek Inc., St. Louis, MO, USA), with additional biochemical tests when indicated. During the second period of the study (period B), bacterial identification was performed by classical phenotypical testing.

The study was approved by the Ethics Committees of all Institutions involved.

2.2. DNA purification and PCR

Isolates identified as *B. cepacia* complex were submitted to DNA extraction as described elsewhere [8], and DNA was quantified in an ultraviolet spectrophotometer (BioPhotometer 6131, Eppendorf AG, Hamburg, Germany).

The genomovar status of the *B. cepacia* complex isolates was identified by sequential PCR targeting specific regions of the *recA* gene as previously described [13–15]. Positive controls consisting of DNA from the *B. cepacia* complex strain belonging to the genomovar tested were included in all reactions.

Virulence markers were identified by PCR targeting the *cblA* (cable pilus) and *esmR* (BCESM) genes as previously described [6,7]. DNA from the *B. cepacia* complex strain AU0355 (epidemic strain expressing both virulence factors) was included in the reactions as positive control.

After amplification, PCR products were visualized by electrophoresis on 1.5% agarose gel stained with 0.5 mg/mL ethidium bromide under UV illumination using an automated system (AlphaImager®, Alpha Innotech Co., San Leandro, California, USA). A negative control consisting of sample without DNA was included in all reactions. All reactions were carried out under a laminar flow UV hood according to standard precautions to prevent PCR carry-over [16].

2.3. DNA sequencing

DNA sequencing of the *recA* gene was performed to confirm the results of the specific PCR. DNA sequencing was carried out using a panel of four primers (BCR 1–4) [13] and BUR1 and BUR2 primers [17], using the Big Dye Terminator kit (Applied Biosystems Incorporation, Foster City, CA, USA) in an automated DNA sequencer (ABI Prism 377, Applied Biosystems). The results obtained were compared to sequences deposited in the Genebank database using the BLAST program.

2.4. Molecular epidemiology (RAPD)

B. cepacia complex isolates were genotyped by the RAPD method using primer 272 (5′-AGCGGGCCAA-3′), as previously described [18]. After amplification, the RAPD products were visualized by electrophoresis on 7.5% acrylamide gel stained with 0.2% silver nitrate. The results were interpreted by direct visualization of the band patterns and isolates which differed by two or more major bands were considered sufficiently divergent to warrant separate strain designations.

3. Results

During the two periods of the study, a total of 672 respiratory samples were obtained from 140 CF patients attending our CF centre. *B. cepacia* complex isolates were identified by culture in 41 samples (35 sputum and 6 oropharyngeal swabs) from 21

Table 1
Genomovar determination by PCR, *recA* gene DNA sequencing and RAPD genotyping of isolates identified as members of the *B. cepacia* complex during the study ($n=32$)

Patient	Isolate	Period of isolation	Genomovar *recA* PCR	Epidemic strain marker (*esmR*)	RAPD typing	DNA sequencing of *recA* gene
B	2	B	IIIA	−	2	*B. cenocepacia*
C	3	B	IIIA	−	3	*B. cenocepacia*
D	4	A	IIIA	−	4	*B. cenocepacia*
D	5	B	IIIA	−	4	*B. cenocepacia*
E	6	A	I	−	5	*B. cepacia*
F	8	B	IIIA	−	7	*B. cenocepacia*
F	9	B	IIIA	−	7	*B. cenocepacia*
F	10	B	IIIA	−	7	*B. cenocepacia*
F	11	B	IIIA	−	7	*B. cenocepacia*
G	12	B	V	−	8	*B. vietnamiensis*
H	13	B	IIIA	−	9	*B. cenocepacia*
H	14	B	IIIA	−	9	*B. cenocepacia*
H	15	B	IIIA	−	9	*B. cenocepacia*
H	16	B	IIIA	−	10	*B. cenocepacia*
I	17	A	V	−	11	*B. vietnamiensis*
K	19	A	IIIB	+	13	*B. cenocepacia*
L	20	A	II	−	14	*B. multivorans*
L	21	A	II	−	NP	*B. multivorans*
L	22	B	II	−	14	*B. multivorans*
L	23	B	II	−	14	*B. multivorans*
L	24	B	II	−	14	*B. multivorans*
L	25	B	II	−	14	*B. multivorans*
M	26	B	II	−	15	*B. multivorans*
N	27	B	IIIA	+	16	*B. cenocepacia*
P	32	A	IIIA	−	18	*B. cenocepacia*
P	33	A	IIIA	−	18	*B. cenocepacia*
Q	34	B	II	−	19	*B. multivorans*
S	36	A	II	−	21	*B. multivorans*
S	37	B	II	−	21	*B. multivorans*
S	38	B	II	−	21	*B. multivorans*
S	39	B	II	−	21	*B. multivorans*
U	41	B	IIIA	−	23	*B. cenocepacia*

NP: not performed.

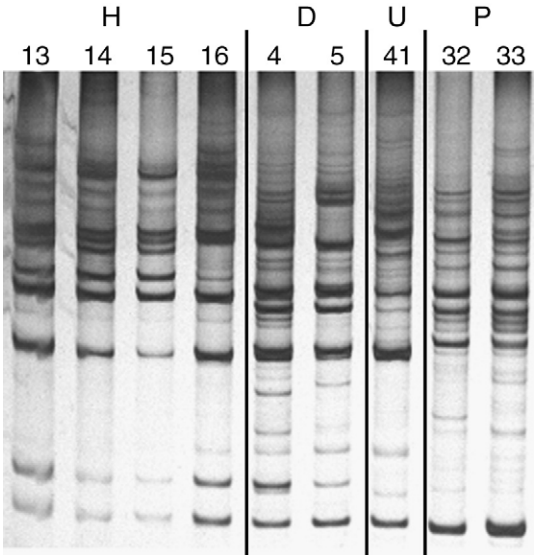

Fig. 1. Genotyping of *Burkholderia cepacia* complex isolates. Acrylamide (7.5%) gel stained with 0.2% silver nitrate. Each cluster of lanes corresponds to isolates (identified by numbers) from one patient, identified by letter (contained in Table 1).

patients. Confirmation of *B. cepacia* complex identity was obtained in 32 isolates (4.7%), from 16 patients (11.4%). *B. cenocepacia* was the most common genomovar identified (17 isolates from 9 patients), followed by *B. multivorans* (12 isolates from 4 patients). Two isolates were identified as *B. vietnamiensis* and one as *B. cepacia* (genomovar I). *B. stabilis*, *B. dolosa* and *B. ambifaria* were not identified among the isolates. All isolates were unambiguously identified. Patients who were positive during the first and second periods kept the same genomovar for as long as 3 years.

DNA sequencing of the *recA* gene confirmed the PCR results of genomovar assignment for all 32 isolates of *B. cepacia* complex. The nine samples with phenotypic misidentification were tested by PCR using BUR1/2 primers and only seven isolates tested positive. After DNA sequencing of these PCR products, isolates were characterized as *Burkholderia gladioli* (5 isolates) and *Xanthomonas campestris* (2 isolates). The other two isolates remained unidentified.

Investigation of virulence markers showed that all isolates were negative for the pilin gene (*cblA*), whereas two isolates were positive for the epidemic strain marker gene (*esmR*), both identified as *B. cenocepacia*.

Genotyping using RAPD detected 17 genotypes among 31 *B. cepacia* complex isolates. One *B. cepacia* complex isolate was not studied (isolate was lost), but it was obtained from patient L who had 5 other isolates, all characterized as *B. multivorans* and showing the same RAPD profile (Table 1). Patients with two or more *B. cepacia* complex isolates during the two study periods conserved the same genotype (Fig. 1) and no identical patterns were identified among different patients. Only one patient presented the last of four isolates of *B. cenocepacia* showing a different genotype (patient H, isolate 16).

4. Discussion

The present study shows that the distribution of genomovars of the *B. cepacia* complex in our Institution is quite similar to that observed elsewhere [19], with a predominance of *B. cenocepacia*, followed by *B. multivorans*. The prevalence of *B. cepacia* complex found was 11.4%, a significantly higher rate than the 2.9% reported for the North American CF population [4]. Data regarding the prevalence of the *B. cepacia* complex in Brazil are scarce, but a recent paper reported a prevalence of 5.2% during a 10-year survey, although the authors did not use selective medium [20].

There is also a lack of information concerning genomovar distribution of *B. cepacia* complex isolates in Brazil, and a well known inconsistent distribution throughout the world. *B. cenocepacia* is the most common genomovar in Canada [21], the United States [22] and Italy [3], but its prevalence may range from 50% in the United States to 80% in Canadian CF centres. However, data from Belgium [23] and France [24] indicate a predominance of infections with *B. multivorans*. In Portugal, recent data indicate a significant increase in the prevalence of *B. cepacia* [25,26], maybe related to contamination of nasal saline solutions [26]. One study combining CF and non-CF *B. cepacia* complex isolates from Recife, Brazil, reported a high prevalence of *B. cenocepacia* (72%) among non-CF isolates, but the genomovar status was determined only in 5/11 CF isolates, with *B. cenocepacia* and *B. vietnamiensis* accounting for 2 isolates each and the other isolate assigned as *B. cepacia* [9].

Microbiological culture identified 41 *B. cepacia* complex isolates but this identity was confirmed in only 32 (78%) isolates. Phenotypic identification of *B. cepacia* complex strains is very difficult due to close similarities among these species and related genera, and misidentifications have therefore been reported [27,28].

Besides that, although *B. cepacia*-selective agar is an excellent culture medium for the isolation of *B. cepacia* complex strains, other similar organisms, such as *B. gladioli* and *Ralstonia* spp., may also grow on this medium. Additional workup of these 9 isolates misidentified by culture was performed by PCR and DNA sequencing resulted positive in seven isolates characterized as *B. gladioli* (5 isolates) and *X. campestris* (2 isolates), while two isolates remained unidentified. These two isolates may warrant future study using 16S rRNA gene sequencing.

Molecular epidemiology using RAPD showed 100% concordance with the results of genomovar determination, and different patients did not share common genotypes. Previous studies have shown that RAPD analysis presents a satisfactory discriminatory power when compared to PFGE [18,29] and its simplicity may favour its routine use for infection control in CF centres.

In the present study, virulence markers were not identified in a high proportion of isolates. This finding is in accordance with the apparent lack of cross-infection among our patients, a situation markedly differing from that observed in other countries such as Canada [21]. A previous Brazilian study of *B. cepacia*

complex isolates has shown a higher prevalence of *esmR* (22/30 isolates) among *B. cenocepacia* isolates, but only two of these isolates were obtained from CF patients and, interestingly, this study also did not find any CF isolate positive for the *cblA* gene [9].

In conclusion, the present study shows that *B. cenocepacia* is the most prevalent member of the *B. cepacia* complex among the CF patients studied here and cross-infection among patients seems unlikely. The prevalence of virulence markers was very low. We also observed that phenotypic misidentifications of *Burkholderia* genus members may occur even when appropriate microbiologic procedures are carefully followed and this finding reinforces the need for the implementation of molecular methods in microbiology laboratories involved in the care of CF patients.

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References

[1] Coenye T, Vandamme P, Govan JR, LiPuma JJ. Taxonomy and identification of the *Burkholderia cepacia* complex. J Clin Microbiol 2001;39(10):3427–36.

[2] Govan JR, Hughes JE, Vandamme P. *Burkholderia cepacia*: medical, taxonomic and ecological issues. J Med Microbiol 1996;45(6):395–407.

[3] Agodi A, Mahenthiralingam E, Barchitta M, Giannino V, Sciacca A, Stefani S. *Burkholderia cepacia* complex infection in Italian patients with cystic fibrosis: prevalence, epidemiology, and genomovar status. J Clin Microbiol 2001;39(8):2891–6.

[4] Cystic Fibrosis Foundation. Cystic fibrosis foundation patient registry annual report 2003. Maryland, USA: Bethesda; 2004.

[5] Coenye T, Vandamme P. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. Environ Microbiol 2003;5(9):719–29.

[6] Sajjan US, Sun L, Goldstein R, Forstner JF. Cable (cbl) type II pili of cystic fibrosis-associated *Burkholderia* (*Pseudomonas*) *cepacia*: nucleotide sequence of the cblA major subunit pilin gene and novel morphology of the assembled appendage fibers. J Bacteriol 1995;177(4):1030–8.

[7] Mahenthiralingam E, Simpson DA, Speert DP. Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. J Clin Microbiol 1997;35(4):808–16.

[8] da Silva Filho LV, Velloso LF, Bento CN, Gytin E, Tateno AF, Levi JE, et al. Use of selective medium for *Burkholderia cepacia* isolation in respiratory samples from cystic fibrosis patients. Rev Inst Med Trop Sao Paulo 2002;44(4):203–8.

[9] Detsika MG, Corkill JE, Magalhaes M, Glendinning KJ, Hart CA, Winstanley C. Molecular typing of, and distribution of genetic markers among, *Burkholderia cepacia* complex isolates from Brazil. J Clin Microbiol 2003;41(9):4148–53.

[10] Carvalho GM, Carvalho AP, Folescu TW, Higa L, Teixeira LM, Plotkowski MC, et al. Transient isolation of *Burkholderia multivorans* and *Burkholderia cenocepacia* from a Brazilian cystic fibrosis patient chronically colonized with *Burkholderia vietnamiensis*. J Cyst Fibros 2005;4(4):267–70.

[11] Silva Filho LV, Levi JE, Bento CN, Rodrigues JC, da Silva Ramos SR. Molecular epidemiology of *Pseudomonas aeruginosa* infections in a cystic fibrosis outpatient clinic. J Med Microbiol 2001;50(3):261–7.

[12] Rosenstein BJ, Cutting GR. The diagnosis of cystic fibrosis: a consensus statement. Cystic Fibrosis Foundation Consensus Panel. J Pediatr 1998;132(4):589–95.

[13] Mahenthiralingam E, Bischof J, Byrne SK, Radomski C, Davies JE, Av-Gay Y, et al. DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* genomovars I and III. J Clin Microbiol 2000;38(9):3165–73.

[14] Vermis K, Coenye T, LiPuma JJ, Mahenthiralingam E, Nelis HJ, Vandamme P. Proposal to accommodate *Burkholderia cepacia* genomovar VI as *Burkholderia dolosa* sp. nov. Int J Syst Evol Microbiol 2004;54(Pt 3):689–91.

[15] Coenye T, Mahenthiralingam E, Henry D, LiPuma JJ, Laevens S, Gillis M, et al. *Burkholderia ambifaria* nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolates. Int J Syst Evol Microbiol 2001;51(Pt 4):1481–90.

[16] Kwok S, Higuchi R. Avoiding false positives with PCR. Nature 1989;339(6221):237–8.

[17] Payne GW, Vandamme P, Morgan SH, LiPuma JJ, Coenye T, Weightman AJ, et al. Development of a recA gene-based identification approach for the entire *Burkholderia* genus. Appl Environ Microbiol 2005;71(7):3917–27.

[18] Mahenthiralingam E, Campbell ME, Henry DA, Speert DP. Epidemiology of *Burkholderia cepacia* infection in patients with cystic fibrosis: analysis by randomly amplified polymorphic DNA fingerprinting. J Clin Microbiol 1996;34(12):2914–20.

[19] LiPuma JJ. Update on the *Burkholderia cepacia* complex. Curr Opin Pulm Med 2005;11(6):528–33.

[20] Alvarez AE, Ribeiro AF, Hessel G, Bertuzzo CS, Ribeiro JD. Cystic fibrosis at a Brazilian center of excellence: clinical and laboratory characteristics of 104 patients and their association with genotype and disease severity. J Pediatr (Rio J) 2004;80(5):371–9.

[21] Speert DP, Henry D, Vandamme P, Corey M, Mahenthiralingam E. Epidemiology of *Burkholderia cepacia* complex in patients with cystic fibrosis, Canada. Emerg Infect Dis 2002;8(2):181–7.

[22] LiPuma JJ, Spilker T, Gill LH, Campbell III PW, Liu L, Mahenthiralingam E. Disproportionate distribution of *Burkholderia cepacia* complex species and transmissibility markers in cystic fibrosis. Am J Respir Crit Care Med 2001;164(1):92–6.

[23] De BK, Malfroot A, Van SL, Lebecque P, Knoop C, Govan JR, et al. Epidemiology of *Burkholderia cepacia* complex colonisation in cystic fibrosis patients. Eur Respir J 2004;23(6):851–6.

[24] Brisse S, Cordevant C, Vandamme P, Bidet P, Loukil C, Chabanon G, et al. Species distribution and ribotype diversity of *Burkholderia cepacia* complex isolates from French patients with cystic fibrosis. J Clin Microbiol 2004;42(10):4824–7.

[25] Cunha MV, Leitao JH, Mahenthiralingam E, Vandamme P, Lito L, Barreto C, et al. Molecular analysis of *Burkholderia cepacia* complex isolates from a Portuguese cystic fibrosis center: a 7-year study. J Clin Microbiol 2003;41(9):4113–20.

[26] Cunha MV, Pinto-de-Oliveira A, Meirinhos-Soares L, Salgado MJ, Melo-Cristino J, Correia S, et al. Exceptionally high representation of *Burkholderia cepacia* among *B. cepacia* complex isolates recovered from the major Portuguese cystic fibrosis center. J Clin Microbiol 2007;45(5):1628–33.

[27] McMenamin JD, Zaccone TM, Coenye T, Vandamme P, LiPuma JJ. Misidentification of *Burkholderia cepacia* in US cystic fibrosis treatment centers: an analysis of 1,051 recent sputum isolates. Chest 2000;117(6):1661–5.

[28] Shelly DB, Spilker T, Gracely EJ, Coenye T, Vandamme P, LiPuma JJ. Utility of commercial systems for identification of *Burkholderia cepacia* complex from cystic fibrosis sputum culture. J Clin Microbiol 2000;38(8):3112–5.

[29] Segonds C, Bingen E, Couetdic G, Mathy S, Brahimi N, Marty N, et al. Genotypic analysis of *Burkholderia cepacia* isolates from 13 French cystic fibrosis centers. J Clin Microbiol 1997;35(8):2055–60.